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Immunostimulatory effects of oriental plum (*Prunus salicina* Lindl.)

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Abstract

The fruit of the plum tree (*Prunus salicina* Lindl.) has been used as a traditional medicinal food in humans to enhance immunity against infectious agents and to treat cancers. However, limited information exists on the mechanisms responsible for its immune enhancing properties. In this study, the immunostimulatory effects of a methanol extract of plum fruit following methanol evaporation and dissolving in PBS were assessed by *in vitro* lymphocyte proliferation, tumor cell cytotoxicity, and nitric oxide (NO) production. The crude methanol extract stimulated spleen lymphocyte proliferation and NO production by cultured macrophages, and inhibited the viability of tumor cells, significantly greater than media controls. Sequential gel filtration chromatographic separation of the extract on Sephadex G-25 and Sephacryl S-200 gel filtration columns resulted in a more purified preparation that retained the ability to induce lymphoproliferation, tumor killing, and NO production. These results suggest that *Prunus*

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salicina contains immunostimulatory components that potentially may be useful in human and veterinary medicine.

Keywords: Plum; Immunostimulation; Lymphocyte; Tumor; Macrophage

Résumé

Le fruit du prunier japonais (*Prunus salicina* Lindl.) a été utilisé comme aliment médicamenteux traditionnel chez l'homme pour augmenter l'immunité contre les agents infectieux et traiter des cancers. Cependant, les informations sur les mécanismes responsables de ses propriétés immunostimulantes sont limitées. Dans cette étude, les effets immunostimulants d'un extrait méthanol de la prune japonaise, obtenu par évaporation du méthanol et dissolution en PBS, ont été évalués par des tests de prolifération lymphocytaire, de cytotoxicité de cellules tumorales, et de production de monoxyde d'azote (NO). L'extrait méthanol brut a stimulé la prolifération des lymphocytes spléniques et la production de NO par les macrophages en culture, et réduit la viabilité de cellules tumorales, de façon significative par rapport aux milieux contrôles. La séparation chromatographique séquentielle sur des colonnes de gel-filtration Sephadex G-25 puis Sephacryl S-200 a produit une préparation plus purifiée qui a conservé la capacité lymphoprolifératives, anti-tumorale, et d'induction de NO. Ces résultats suggèrent que les prunes japonaises contiennent des composants immunostimulants qui pourraient être potentiellement utiles en médecine humaine et vétérinaire.

Mots clés : Prune ; Immunostimulation ; Lymphocyte ; Tumeur ; Macrophage

1. Introduction

Plum (*Prunus salicina* Lindl.) has been cultivated and propagated since ancient times and is considered to be a healthy food because of its well-known mild laxative effect [1]. Several studies have investigated the chemical constituents of plum that are responsible for its biological effects on human health. For example, the laxative action of plum has been attributed to its high fiber, sorbitol, and phenolic contents [2]. Phenolic compounds in plum may delay glucose absorption [3] and inhibit the oxidation of low density lipoproteins *in vitro* [4], and thus might serve as preventive agents against chronic illnesses such as diabetes, heart disease, and cancer. In other fruits, such as strawberry and mulberry, total phenolic content was significantly correlated with *in vitro* splenocyte proliferation [5]. Phenolic components of the *Terminalia chebula* Retz. fruit, commonly known as black myroblans, inhibited the growth of human (MCF-7, HOS-1, PC-3, PNT1A) and mouse (S115) cancer cells *in vitro* [6]. Finally, polyphenols isolated from blueberry and cranberries were found to protect endothelial cells against oxidative (H_2O_2) and inflammatory (TNF-α) insults [7].

Recent interest in the nutrition-based enhancement of innate immunity in humans has focused on exploring the potential immune-stimulating properties of naturally occurring dietary substances. Dandelion leaf and some mushrooms were shown to stimulate the proliferation of lymphocytes, activate macrophages, and inhibit the growth of tumor cells [8–10]. Similar to strawberry and mulberry, plum also possesses high phenolic content,

which shows a significant positive correlation with splenocyte proliferation [5]. Plum has been traditionally used in Asia as a medicinal food to enhance immunity and treat cancers. However, in spite of its well-known medicinal properties, few studies have characterized the effects of plum on immunity. Therefore, the current investigation was undertaken by choosing the general methods used to examine stimulation of the chicken immune system to verify the potential immunoenhancing properties of plum.

2. Materials and methods

2.1. Preparation of methanol extract

Plum (*Prunus salicina* Lindl.), maintained by a proper chain of custody and authenticated by a botanist, was supplied by National Rural Resources Development Institute (Suwon, South Korea). Seeds were removed after washing by flowing water and distilled water, freeze-dried, powdered and stored at −80 °C until use. Methanol extraction was carried out by adding 100 ml of 80% methanol to 30 g of plum powder with vigorous shaking for 48 h at room temperature. The process was repeated three times, the combined extracts were rotary evaporated (EYELA, Irvine, CA), and the residue was freeze-dried and stored at −80 °C until use. Prior to use, the extract was dissolved in deionized water or PBS, pH 7.2 as a 1.0 mg/ml stock solution, sterilized by membrane filtration through a 0.45 μm filter and subsequent dilutions in PBS were made.

2.2. Chromatographic fractionation of plum methanol extract

The dried plum extract was dissolved in deionized water, centrifuged at 5000 rpm for 15 min, and the soluble portion was applied to a Sephadex G-25 column (1.6 cm × 2.5 cm). The material eluting in the void volume was collected, sterile filtered, and freeze-dried. This extract was dissolved in deionized water and separated on a HiPrep Sephacryl S-200 column (16 cm × 60 cm) in deionized water using the AKTA-FPLC system (GE Healthcare, Piscataway, NJ). During this process, eight 5.0 ml fractions were obtained (F 1, F 2, F 3, F 4, F 5, F 6, F 7, and F 8) and assayed for lymphocyte proliferation, tumor killing, and NO production as described below. Fraction 2 (F 2), showing the greatest bioactivity, was refractionated on the S-200 column to obtain fraction II (F II), which was freeze-dried, dissolved in PBS, and sterile filtered prior to assay. Acquisition and treatment of the chromatographic data were carried out using UNICORN software (GE Healthcare) and absorbance at 280 nm was measured for peak detection and quantification.

2.3. Experimental animals

Specific pathogen-free White Leghorn inbred chickens (Hy-vac, Adel, IA) at 3 weeks of age were used in this study. All experiments were approved by the Animal and Natural Resources Institute IACUC.

2.4. Immunomodulating activity

2.4.1. Spleen lymphocyte proliferation assay

Spleens were removed and placed in a Petri dish with 10 ml of Hanks' balanced salt solution supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma, St. Louis, MO). Single cell suspensions were prepared and lymphocyte proliferation was carried out as described [10–13]. Briefly, isolated splenocytes were adjusted to 1×10^7 cells/ml in enriched RPMI-1640 medium without phenol red (Sigma) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Splenocytes (100 μl/well) were cultured with 100 μl of the plum methanol extract at 62.5 μg/ml (w/v), individual chromatography fractions, or 0.25 μg/ml concanavalin A (Con A, Sigma) as a positive control in 96-well flat bottom plates. The eight fractions from the first S-200 column were tested undiluted and fraction F II from the second S-200 separation was tested at 2.0, 4.0, or 8.0 μg/ml (w/v). The cells were incubated at 41 °C in a humidified incubator (Forma, Marietta, OH) with 5% CO_2 for 48 h and cell number was measured using WST-8 (Cell-Counting Kit-8®, Dojindo Molecular Technologies, Gaithersburg, MD) or [^{3}H]-thymidine uptake as described [11,14]. For the WST-8 assay, optical densities were measured at 450 nm using a microplate spectrophotometer (BioRad, Hercules, CA). For the thymidine uptake assay, the cells were radiolabeled for 4 h with 0.25 μCi/well of [^{3}H]-thymidine (Perkin-Elmer Life Science, Boston, MA), harvested using a semi-automated cell harvester (Tomtec, Orange, CT), and radioactivity was determined by liquid scintillation counting (1450 Microbeta Wallac Trilux, Perkin-Elmer Life Sciences, Waltham, MA).

2.4.2. Inhibition of tumor cell growth

RP9 tumor cells [15] were cultured at 1×10^6 cells/ml (100 μl/well) with 100 μl of plum crude methanol extract (62.5, 125, 250, or 500 μg/ml), its chromatography fractions, or 2.0 μg/ml recombinant tumor necrosis factor-α (TNF-α, R&D Systems, Minneapolis, MN) or 12.5 μg/ml lipopolysaccharide-induced TNF-α factor (LITAF) [16] as positive controls in 96-well microtiter plates at 41 °C in a humidified incubator supplemented with 5% CO_2 for 48 h. Cell viability was measured using [^{3}H]-thymidine or WST-8 as described above.

2.4.3. Nitric oxide (NO) production by macrophages

HD11 macrophages [10,17] were cultured at 1×10^7 cells/ml (100 μl/well) with 100 μl of plum crude extract (62.5, 125, 250, or 500 μg/ml), its chromatography fractions, or recombinant interferon-γ (IFN-γ) (1.0 μg/ml) in 96-well microtiter plates in a humidified incubator at 41 °C and 5% CO_2 for 24 h. Following incubation, 100 μl of cell culture supernatants were transferred to fresh flat-bottom 96-well plates, mixed with 100 μl of Griess reagent (Sigma) and the plates were incubated for 15 min at room temperature. The optical densities were measured at 540 nm and nitrite concentration was determined using a standard curve generated with known concentrations of sodium nitrite.

2.5. Statistical analyses

Data analyses were performed using SPSS software (SPSS 12.0 K for Windows). All data were expressed as means ± S.E.M. values. The ANOVA test was used to test for

differences between the groups. Duncan's multiple range test was used to analyze differences between the mean values and differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Spleen lymphoproliferation

The crude methanol extract of plum powder, when tested at 62.5 μg/ml, stimulated splenocyte proliferation at a significantly higher level compared with the medium only control (Fig. 1A). When the crude plum extract was resolved by Sephadex G-25 and Sephacryl S-200 chromatographic columns, all of the fractions exhibited lymphoproliferative activity greater than the medium control, although fraction F 2 showed the greatest

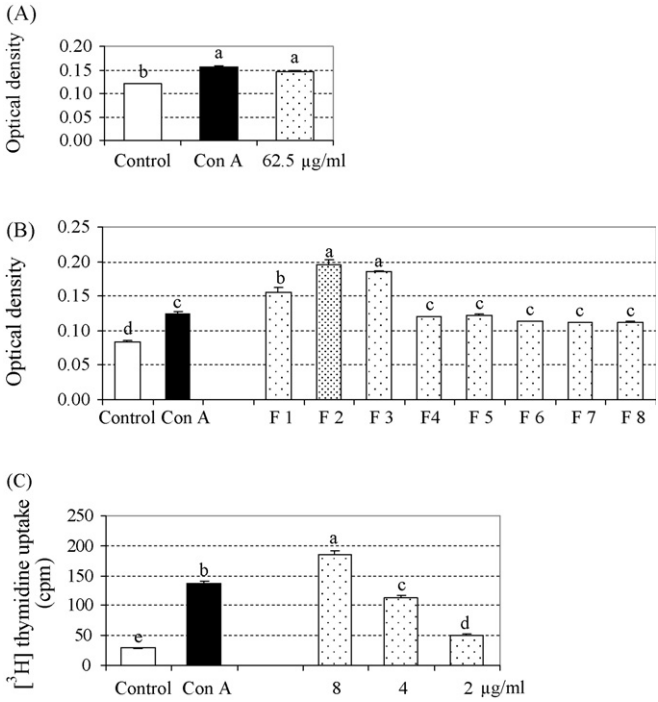

Fig. 1. Effects of crude and fractionated extracts of plum on spleen lymphocyte proliferation. (A) Crude methanol extract of plum tested at 62.5 μg/ml and compared with medium alone negative control and 0.25 μg/ml Con A positive control. (B) The first eight fractions (F 1–F 8) obtained from crude extract during the first separation were tested undiluted and compared with negative and positive controls. (C) F II obtained from F 2 during the second separation tested at the indicated concentrations and compared with negative and positive controls. Cell numbers were measured by the WST-8 assay (A and B) or [^{3}H]-thymidine uptake (C) as described in Section 2. Each bar represents the mean ± S.E.M. values obtained from triplicate culture wells from three chicken spleens. Within each graph, bars not sharing the indicated letters are significantly different ($P < 0.05$) according to Duncan's multiple range test.

activity (Fig. 1B), being greater than that induced by the positive control, 0.25 μg/ml of Con A. Fraction F 2 was refractionated by Sephacryl S-200 chromatography resulting in a single A_{280} peak (fraction F II). Fraction F II induced dose-dependent lymphoproliferation that was significantly greater than the medium control (Fig. 1C). No toxic effect of the methanol extract or column fractions on spleen cells was observed at any of the concentrations tested.

3.2. Tumor cell viability

The crude plum extract significantly decreased the viability of RP9 tumor cells compared with the medium control (Fig. 2A). Its effect on RP9 tumor cells at all concentrations tested was similar to that of the positive control, 2.0 μg/ml of human

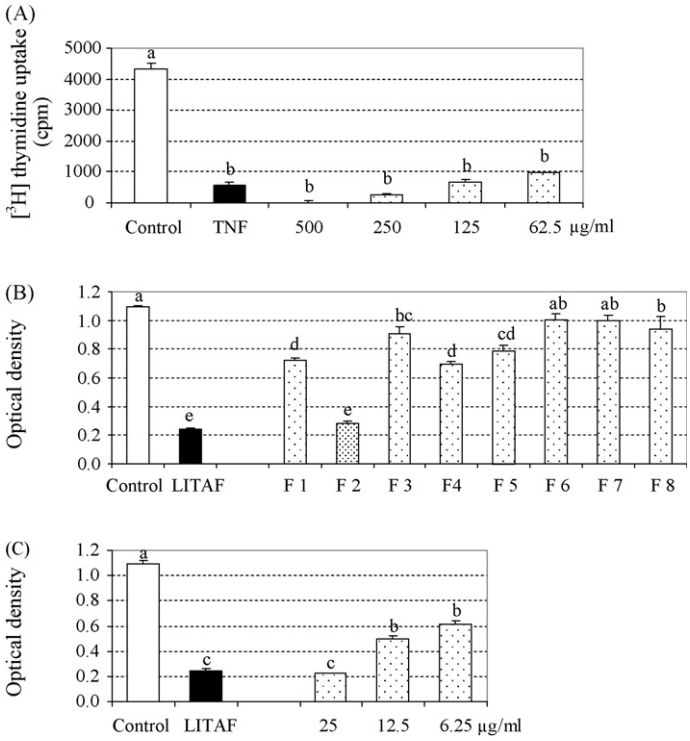

Fig. 2. Effects of crude and fractionated extracts of plum on RP9 tumor cell viability. (A) Crude methanol extract of plum tested at the indicated concentrations and compared with medium alone negative control and 2.0 μg/ml recombinant TNF-α positive control. (B) The first eight fractions (F 1–F 8) obtained from crude extract during the first separation tested undiluted and compared with medium negative control and 12.5 μg/ml recombinant chicken LITAF positive control. (C) F II obtained from F 2 during the second separation tested at the indicated concentrations and compared with negative and positive controls. Cell numbers were measured by [^{3}H]-thymidine uptake (A) or the WST-8 assay (B and C) as described in the Materials and methods. Each bar represents the mean ± S.E.M. values obtained from triplicate culture wells. Within each graph, bars not sharing the indicated letters are significantly different ($P < 0.05$) according to Duncan's multiple range test.

recombinant TNF-α. As shown in Fig. 2B, six peaks of cytotoxic activity were observed among eight peaks obtained from the crude plum extract during the first S-200 separation. Based on the fact that the peak in lymphoproliferative activity also eluted in fraction F 2 (Fig. 2B), this fraction was further resolved by Sephacryl S-200 chromatography for tumor cytotoxic analysis. The resulting F II fraction was observed to possess cytotoxic activity against RP9 cells, with the highest concentration tested (25 μg/ml) displaying cytotoxicity equivalent to that of the positive control, 12.5 μg/ml of LITAF (Fig. 2C).

3.3. NO production

The crude plum methanol extract stimulated NO production by HD11 macrophages in a dose-dependent manner at a significantly greater level compared with the medium control (Fig. 3A). The highest concentration tested (500 μg/ml) exhibited greater bioactivity than the positive control, 1.0 μg/ml of IFN-γ. Unlike the activity stimulating lymphocyte proliferation or inhibiting tumor cell viability, however, no clear peak in NO production was observed by Sephacryl S-200 chromatography, and all eight fractions produced greater

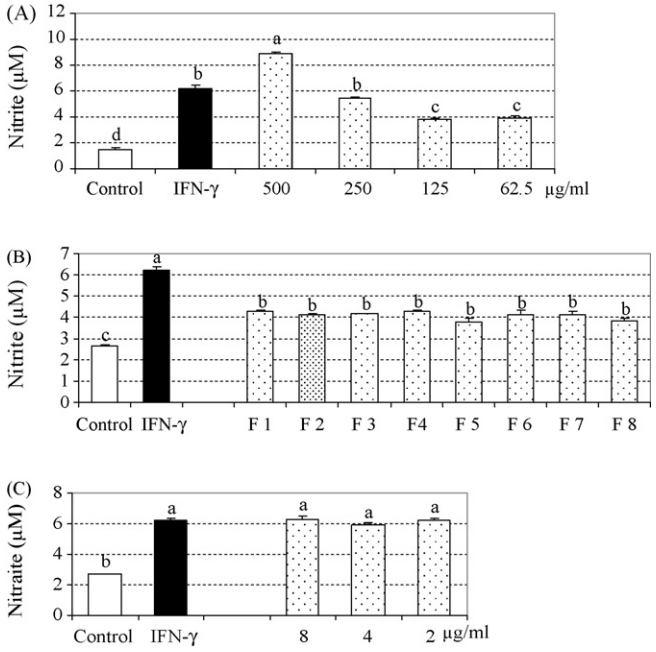

Fig. 3. Effects of crude and fractionated extracts of plum on NO production by HD11 macrophages. (A) Crude methanol extract of plum tested at the indicated concentrations and compared with medium alone negative control and 1.0 μg/ml recombinant IFN-γ positive control. (B) The first eight fractions (F 1–F 8) obtained from crude extract during the first separation tested undiluted and compared with negative and positive controls. (C) F II obtained from F 2 during the second separation tested at the indicated concentrations and compared with negative and positive controls. NO levels were measured as described in Section 2. Each bar represents the mean ± S.E.M. values obtained from triplicate culture wells. Within each graph, bars not sharing the indicated letters are significantly different ($P < 0.05$) according to Duncan's multiple range test.

NO production compared with the medium control (Fig. 3B). The activity of fraction F II obtained from the second fractionation of F 2 following Sephacryl S-200 fractionation was comparable to that of chicken recombinant IFN-γ (1.0 μg/ml) at all concentrations tested (Fig. 3C).

4. Discussion

In this study, we showed that a methanol extract of plum, used as a traditional medicinal plant in humans to enhance resistance to acute and chronic diseases, is highly immunostimulatory. When compared with the medium control, the methanol extract significantly increased the spontaneous proliferation of spleen lymphocytes without noticeable toxicity at 62.5 μg/ml. A single fraction (F II) isolated by sequential gel filtration chromatographic separations showed significantly higher splenocyte proliferation, even at the lowest tested concentration of 2.0 μg/ml, when compared with the medium control.

Lymphocytes participate principally in innate (monocytes and NK cells) and acquired (T and B cells) immune defenses, and previous studies have demonstrated that the effects of natural food and herbal products on host defense against microbial pathogens and tumors were directly correlated with their ability to stimulate lymphocyte proliferation [8–10,18–21]. Splenocyte proliferation stimulated by medicinal fruits and vegetables has been attributed to their high concentration of phenolic compounds. Although the oriental plum was shown to contain a relatively high total phenolic content among a group of 13 selected fruits and vegetables recently examined by Lin and Tang [5], the correlation between plum phenolic level and mouse splenocyte proliferation was relatively weak. The difference in proliferation that we observed compared with that of the prior study [5] might be related to the different animal system employed, and/or different subspecies of *Prunus salicina* utilized.

We observed that the crude extract of plum inhibited the viability of a tumor cell line in a dose-dependent manner, and that the more purified fraction F II showed significantly reduced viability of RP9 tumor cells even at the lowest tested concentration of 6.25 μg/ml. The tumor cell inhibitory activity of this partially purified fraction at 25 μg/ml was similar to that of LITAF, a molecule with known anti-tumor properties [16]. The extracts may exert both direct (on tumor cells themselves) and indirect (potentiate immune cells to kill tumor cells) anti-tumor activities. These results seem to suggest that a pharmacologic basis exists for the historical use of plum as a medicinal food to treat human cancers.

Macrophages play a significant role in host defense against infectious agents and tumors, in part, through the elaboration of effecter molecules such as NO [22] and increased NO production by IFN-γ has been reported [23]. The current study demonstrates that the methanol extract of plum, both the crude extract and chromatographic fractions thereof, significantly stimulated NO production by chicken macrophages, comparable to that of the IFN-γ positive control. These results corroborate several previous reports demonstrating that the bioactive properties of several different medicinal foods and herbs were affected through macrophage activation [24–27].

Macrophages and T lymphocytes produce immunoregulatory cytokines and chemokines during protective immune responses [28]. IFN-γ is produced by natural killer cells

and T lymphocytes and stimulates macrophages to secrete nitric oxide (NO), which forms peroxynitrite, a potent oxidant with anti-microbial properties [29,30]. Various experimental strategies to enhance host protective immunity to microbial pathogens using pro-inflammatory cytokines, such as IFN-γ, have been reported [31,32]. Early cellular immune responses characterized by IFN-γ production are critical to effective protective responses against infectious diseases [31]. Our results in this study show the stimulating effect of plum extract on macrophage NO production and splenocyte proliferation. This may be related to the result in our previous *in vivo* study where plum powder increased splenocyte proliferation and IFN-γ expression in chickens [33].

In summary, the results from this study provide a preliminary rationale basis for further analysis of the immunostimulating effects of plum. Future *in vitro* and *in vivo* studies are necessary to identify the chemical compounds present in the plum fruit that account for its demonstrated effects, and to delineate the cellular and molecular mechanisms responsible for the enhancement of immunity.

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